Molecular diagnosis of Myxomavirus and characterization of strains detected in Italy during 2010-2011

Cavadini P.[1], Lavazza A.[2], Botti G.[1], Brivio R.[3], Capucci L.[1]

[1]Department of Genomics, IZSLER, Italy
[2]Department of Virology, IZSLER, Italy
[3]Servizio SATA, APA Regione Lombardia, Italy

Corresponding Author: Patrizia Cavadini, Department of Genomics, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna "Bruno Ubertini" Italy - Tel. +39 0302290373 - Fax: +39 0302290559 - Email: patrizia.cavadini@izsler.it

ABSTRACT: Myxomatosis, a lethal systemic disease of European rabbits (*Oryctolagus cuniculus*), is caused by Myxoma Virus (MV). The entire genome of the MV has been sequenced, allowing a systemic survey of the functions of a large number of putative pathogenic factors. The data present in literature have shown that myxoma virus is characterized by a high degree of genetic stability. Aim of this study was to identify molecular markers, on MV genome, for characterizing the isolates circulating in Italy during epidemiological studies. As a first step a PCR-based assay was established to rapidly diagnose the presence of myxoma virus in biological samples isolated from naturally infected rabbits. Twelve isolates collected during 2010-2011 from different Italian regions (wide ranging geographic locations), were then partially sequenced. Based on the sequence analysis we found nucleotides substitutions, deletions and insertions randomly present on the MV genome. We identified at least 6 different strains circulating in Italy during that time. In particular, one strain was identified in 5 samples (Cluster 1), another one in 3 samples (Cluster 2) and the remaining strains in single samples. Since three point mutations common to Cluster 1 can be identified with PCR-RFLPs analysis, these markers could be easily used to perform epidemiologic studies. Interestingly, one strain from Sicily region (South Italy) has shown 100% nt identity with the Borghi vaccine strain, with a region of the genome suggesting a possible *in vivo* recombination between a circulating wild virus and a Borghi vaccine strain.

Key words: Myxoma virus, Vaccines, PCR, Genotyping.

INTRODUCTION – Myxomatosis, a lethal systemic disease of European rabbits (*Oryctolagus cuniculus*), is caused by Myxoma Virus (MV) (Fenner, 1959). Depending from the clinical form, the virus may be passively transmitted from skin lesions of diseased rabbits by biting arthropods (nodular, "classical" form) or by contamination of conjunctivae or nasal passages by infectious discharges, among rabbits in direct contact (amyxomatous "respiratory" form).
The entire genome of the myxoma virus Lausanne has been sequenced (Cameron *et al.*, 1999; Kerr *et al.*, 2012) and partial characterization at the molecular level of field isolates was done (Dalton *et al.*, 2009; Morales *et al.*, 2008). Recently a complete study of evolution and attenuation of MV in Australia and Europe has been performed (Kerr *et al.*, 2012). The genome is 161.8Kb coding 171 genes, 12 of that are duplicated in the terminal inverted repeat regions (TIR/L an TIR/R). The genes localized in the central

portion of the genome have structural functions, whereas the genes close or inside the TIRs have immunomodulatory functions. In Italy, industrial rabbits are mostly vaccinated against myxomatosis using the attenuated myxoma virus strains Borghi or SG33, the latter completely sequenced (Cavadini *et al.*, 2010; Camus-Bouclainville *et al.*, 2011).

MATERIALS AND METHODS – Rabbit kidney epithelial cells (RK13) were infected with Cunivax myxoma vaccine (Borghi strain), Dervaximyxo SG33© vaccine and Moses strain (SLS), subjected to one freeze-thawing passages and 100µl of cryolysates incubated with the lysis buffer for DNA extraction. The viral DNA was also extracted from tissues of 12 samples collected during 2010-2011 in Italy (Table 1). Approximately 5ng of extracted DNA was amplified by PCR, and the amplification products submitted to sequence analysis. Nineteen PCR products were sequenced, spanning approximately 20% of the entire genome (30 ORFs sequenced).

Table 1 – Samples analyzed in this study

Collection year	Town (Province)	Organ
2010_1	Cento (FE)	Lung
2010_2	Reggio nell'Emilia (RE)	Skin
2010_3	Cremeno (CO)	Skin
2010_4	Catania (CT)	Skin
2010_5	Crema (CR)	Skin
2010_6	Albano Sant'Alessandro (BG)	Skin
2011_1	Brescia (BS)	Viscera
2011_2	Volta Mantovana (MN)	Skin
2011_3	Pozzolengo (BS)	Eylid
2011_4	Piacenza (PC)	Eylid
2011_5	Sant'Angelo Muxaro (AG)	Viscera
2011_6	Offlaga (BS)	Skin

RESULTS AND CONCLUSIONS – The sequence information obtained from the 12 strains was compared to the reference strain “Lausanne” (G.B.: NC_001132.2). The genomic regions analyzed were chosen based on the following criteria: (1) M005–M002, M017–M018, M036, M088 and M134 regions, because mutations were previously described in field strains; (2) M022 and M071 encode, respectively, the major envelope protein and an immunodominant envelope protein, exposed to the immune system of the host; (3) M114–M121 genes are conserved within poxviruses; (4) the M129–M131 region includes the M130 gene encoding for a virulence factor; (5) the M142–M144 region includes the M143 gene encoding for an apoptosis regulator and (6) the M150–M152 and M153–M156 regions include immune modulators (M150, M152, M153) and the anti-apoptotic factor Serp2 (M151). In total 23 nucleotide substitutions and 12 indels were found. The distribution of the mutations is random and not localized in specific regions, as already described in literature. The indels are mainly localized in the TIRs regions or in the intergenic regions rich of stretch of repeated sequences. Based on the detected mutations, we have identified at least 6 different strains circulating in Italy during 2010-2011, characterized by specific mutations. In particular, one viral strain (identified as Cluster 1 of mutations) was identified in 5 samples and another one (Cluster 2) in 3 samples. Because three point

mutations common to Cluster 1 can be identified with PCR-RFLPs analysis (the mutations introduce or remove respectively the restriction enzymes *AflIII*, *ScaI* and *BspHI*), these markers could be useful to perform epidemiologic studies. The RFLP-ScaI has been already checked on 47 samples collected from 2010-2012 and 33 resulted positive for the presence of this polymorphism. This partial molecular characterization suggests that the viral strain belonging to Cluster 1 is the most representative. The remaining strains are characterized by mutations that cannot be analyzed by PCR-RFLPs and consequently specific PCR and/or multiplex-PCR will be set up. Interestingly, one strain from Sicily region (South Italy) has shown 100% nt identity with the Borghi vaccine strain with a portion of the genome suggesting a possible *in vivo* recombination between a circulating virus and the Borghi vaccine strain.

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